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SURVIVAL OF MICROBIAL PATHOGENS IN THE MARINE ENVIRONMENT.(U)
MAY 78 R R COLWELL, F M HETRICK N00014-76-C-0405
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Survival studies in filtered seawater at varying temperatures showed an increase in parvovirus viability with decreasing temperature. In further investigation of enterovirus elimination from untreated seawater, a marine bacterium, Acinetobacter calcoaceticus, was isolated and found to possess a poliovirus I, non-filterable, virucidal effect. Survival of coliforms and human enteric pathogens in Chesapeake Bay also has been examined.

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Contract Title: SURVIVAL OF MICROBIAL PATHOGENS IN THE MARINE ENVIRONMENT

Contract No.: N00014-76-C-0405

Principal Investigators: Dr. R. R. Colwell
Dr. R. M. Hetrick

Assisted by: Mr. J. Gilbert
Mr. J. Hoffman

Period Covered. January 1, 1977 - December 31, 1977

METHODS

Viruses Employed

Stocks of poliovirus type 1 (Mahoney strain), Coxsackie B-5 (Faulkner strain) and ECHO 6 (D'Amori strain) viruses were all prepared in a continuous line of monkey kidney cells (BGM line). Stocks of the human parvovirus H-1 were propagated in rat embryo (R.E.) cells which were obtained as primary cultures from Microbiological Associates, Bethesda, MD. All cells were routinely propagated with Eagle's Minimum Essential medium prepared with Earle's salts (EMEM) and supplemented with 10% fetal calf serum.

Virus Assay Procedure

The following procedure was used for the 3 enteroviruses. Confluent BGM cultures, grown in 60 x 15 mm culture dishes, were infected with serial 10-fold dilutions of the virus. Following a 30 min. adsorption period, 5 ml of an overlay medium consisting of equal volumes of 1.2% agarose and 2X EMEM with 4% fetal calf serum were added. The cultures were incubated for 48 hr (polio) or 72 hr (ECHO-6 and Coxsackie B-5) at 35°C in 5% CO_2. The cells were then fixed with formalin (40%) for 1 hr, the agarose and formalin were decanted, and the cell sheet stained with 1% methylene blue for 10 min. Plaques were counted and the results are expressed as the number of pfu's per ml.

For H-1 virus, virus dilutions were added to wells in microtiter plates which contained freshly seeded R.E. cells. The plates were incubated at 35°C in an atmosphere of 5% CO_2. Wells were examined daily for cytopathic effects and the $TCID_{50}$ was determined after 10 days of incubation.

Bacterial Assay Procedure

For *Acinetobacter calcoaceticus*, serial 10-fold dilutions were made in ocean water sterilized by filtration (0.22 μM Millipore membrane). The

dilutions (0.1 ml volumes) were spread on marine salts agar containing yeast extract (Difco-2216). Plates were incubated at room temperature for 48 hr. Results are expressed as the number of colony forming units (cfus) per ml.

RESULTS

1. Effect of temperature on parvovirus survival in seawater.

The parvoviruses are among the hardiest of the animal viruses with stability characteristics very similar to those of infectious hepatitis virus. One of the human parvoviruses, H-1 virus, was selected as a representative of the group.

Procedure

Atlantic ocean coastal water (salinity 28 parts per thousand, pH 7.6) was seeded with H-1 virus to contain approximately 10^4 $TCID_{50}$'s per ml. Four replicate flasks were employed, one for incubation in a shaker bath at each of the following temperatures: 4°C, 15°C, 25°C, and 37°C. Samples were taken immediately after adding the virus (Time 0) to each flask and at selected weekly intervals thereafter. Samples were either assayed at the time of collection or they were stored frozen until cell cultures were ready. No drop in titer was noted between freshly assayed samples and the samples assayed after 9 months of storage at -70°C.

Results

As observed in our previous studies with enteroviruses, a decrease in survival time was seen with increasing temperatures of incubation. Little or no drop in infectivity was seen at 4°C (Figure 1) or 15°C (Figure 2) during the first 48 weeks of incubation. At 37°C (Figure 4) and 25°C (Figure 3), viral infectivity was lost within 10 and 14 weeks respectively however when compared to the enteroviruses, H-1 virus is more stable in

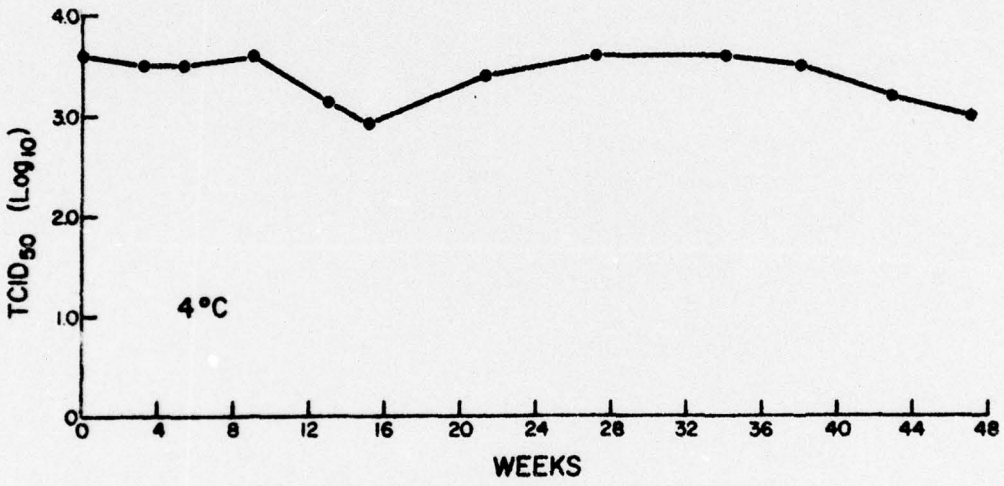

FIG. 1. Survival of H-1 Parvovirus in Seawater at 4°C.

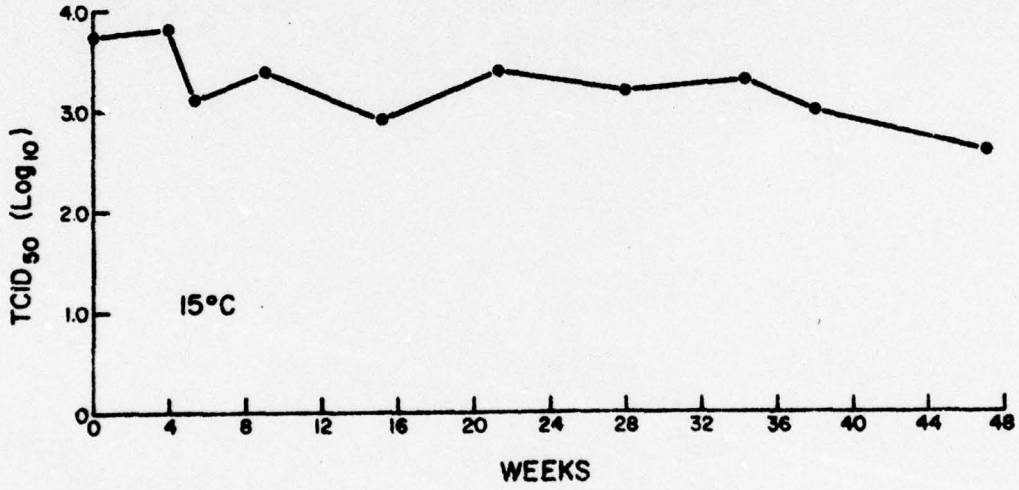

FIG. 2. Survival of H-1 Parvovirus in Seawater at 15°C.

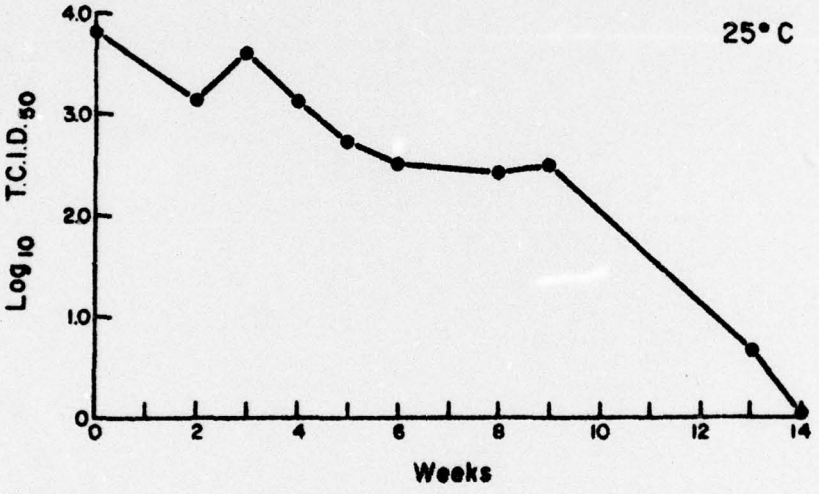

FIG. 3. Survival of H-1 Parvovirus in Seawater at 25°C.

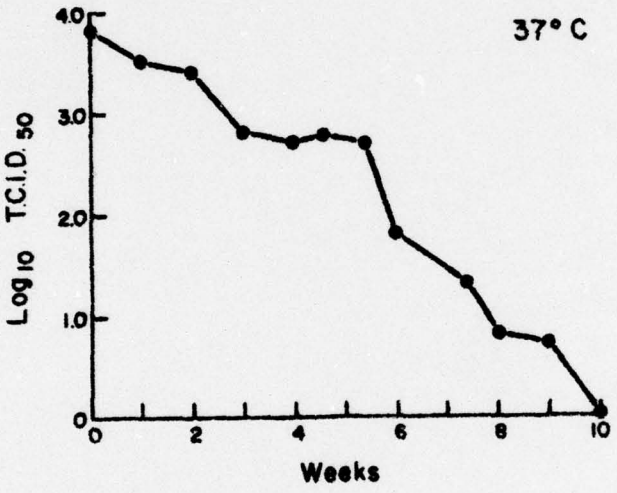

FIG. 4. Survival of H-1 Parvovirus in Seawater at 37°C.

seawater as all 3 enteroviruses were inactivated within a week at 37°C and with 7-10 weeks at 25°C.

2. Studies of the virucidal property of seawater.

Methods

In attempting to isolate virucidal properties, Poliovirus 1 was added to a flask of 100 ml of untreated ocean water (test flask) and to a flask of 100 ml of filtered (0.22 μM Millipore membrane) ocean water (control flask). The flasks were then incubated at 25°C with samples being taken daily for virus assay. When a significant drop in virus titer was noted in the test flask as compared to the control flasks, samples of the test flask were plated on marine salts agar. Different bacterial types were picked from the plate, transferred to marine salts broth and allowed to grow for 48 hr at 25°C. Dilutions of the broth suspension were then tested for virucidal activity against poliovirus suspended in filtered ocean water. The remaining broth cultures of bacteria were made to 50% by volume with glycerol and stored for future use.

Results

Poliovirus 1 suspended in untreated seawater underwent a 5 log drop in titer within 3 days and infectivity was undetectable after 8 days. In contrast, poliovirus suspended in filtered seawater showed only a 1 log drop in titer after 4 days and a 2 log drop after 8 days. Pure cultures of bacteria were isolated from the flask of untreated seawater and tested for viral inactivating properties (Figure 5). Within 5 days, a 5.5 log drop in poliovirus titer had occurred in one flask (D). The other 4 bacterial isolates had no apparent activity and the drop in titer noted (2-3 logs) was similar to that of the control.

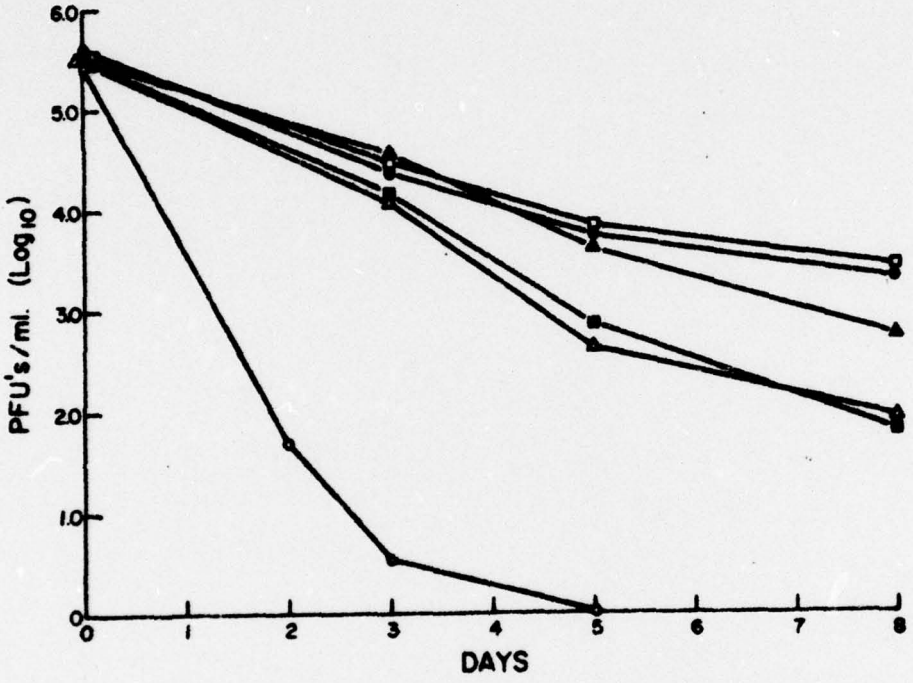

FIG. 5. Survival of Poliomyelitis Virus in the Presence of Bacterial Isolates.

O - Presence of Acinetobacter calcoaceticus

□ - Presence of Bacterial isolate A

Δ - Presence of " " B

▲ - Presence of " " C

■ - Presence of " " E

● - Seawater with no bacteria present

Upon retesting of this isolate for poliovirus inactivation, a 5 log drop in viral infectivity was obtained in 6 days while the control flask showed only a 1.5 log drop after 10 days (Figure 6). Growth of the bacterium was also assayed during this time and it can be seen in Figure 7 that the increase in bacterial numbers correlates well with the drop in viral titer.

The active bacterial isolate was found to be a gram, motile rod that is oxidase - and catalase +. Growth occurred at 4°C and was dependent on marine salinity. The isolate possesses multiple antibiotic resistance and is tentatively identified as Acinetobacter calcoaceticus. Tests were conducted to determine if this organism possessed virucidal activity against the other viruses. No significant drop in titer was noted with either Coxsackie B-5 virus (Figure 8) or H-1 virus (Figure 9) when compared to the control flasks however a 5.2 log drop in titer was seen with ECHO-6 (Figure 10) after 10 days during which time the drop was only 2.2 logs in the control flask. Preliminary attempts have been made to determine if the viral inactivation process is due to some activity on the part of the bacterial cells themselves or whether it is due to some metabolic product.

Filtrates of A. calcoaceticus suspensions taken at 1 day intervals were not found to possess any virucidal properties (Figure 11).

Other studies

Although peripheral to the main objectives of the contract work, studies were initiated to determine if lymphocyte cell cultures might provide any advantages to us over the standard monolayer assays for parvoviruses. This work was conducted by a graduate student in my laboratory, Leslie Bass, and although she received no salary from the contract some supplies were provided for her work. Two publications have resulted form this work and ONR support is acknowledged in both.

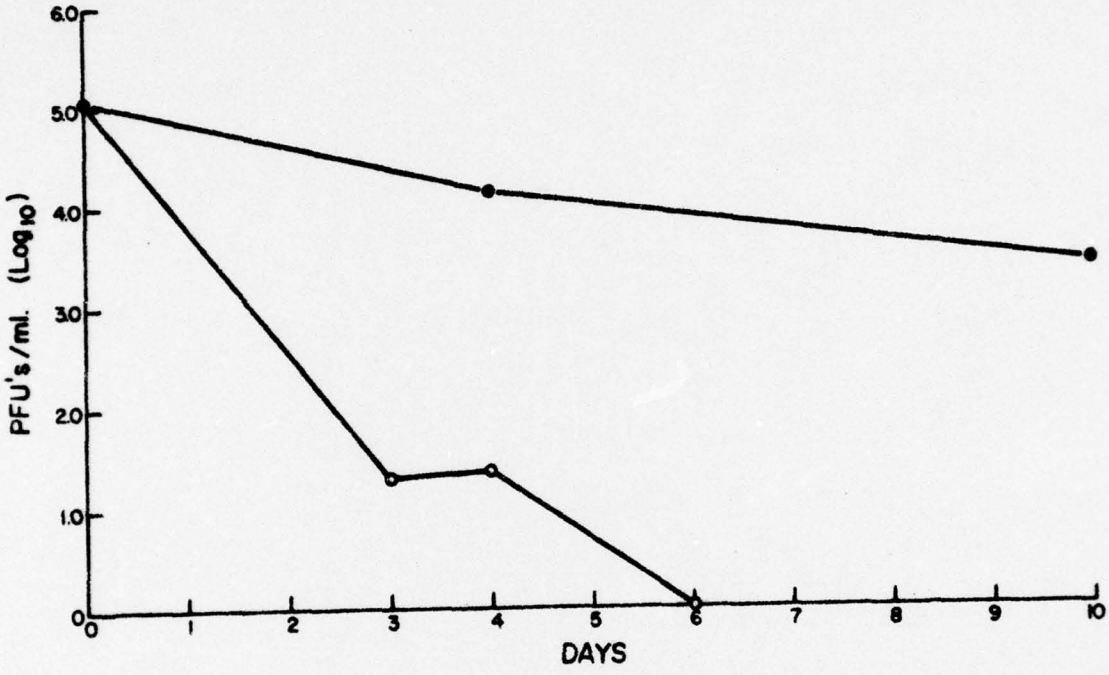

FIG. 6. Inactivation of Poliomyelitis Virus by A. calcoaceticus.

O - A. calcoaceticus present

● - Poliovirus I in seawater alone

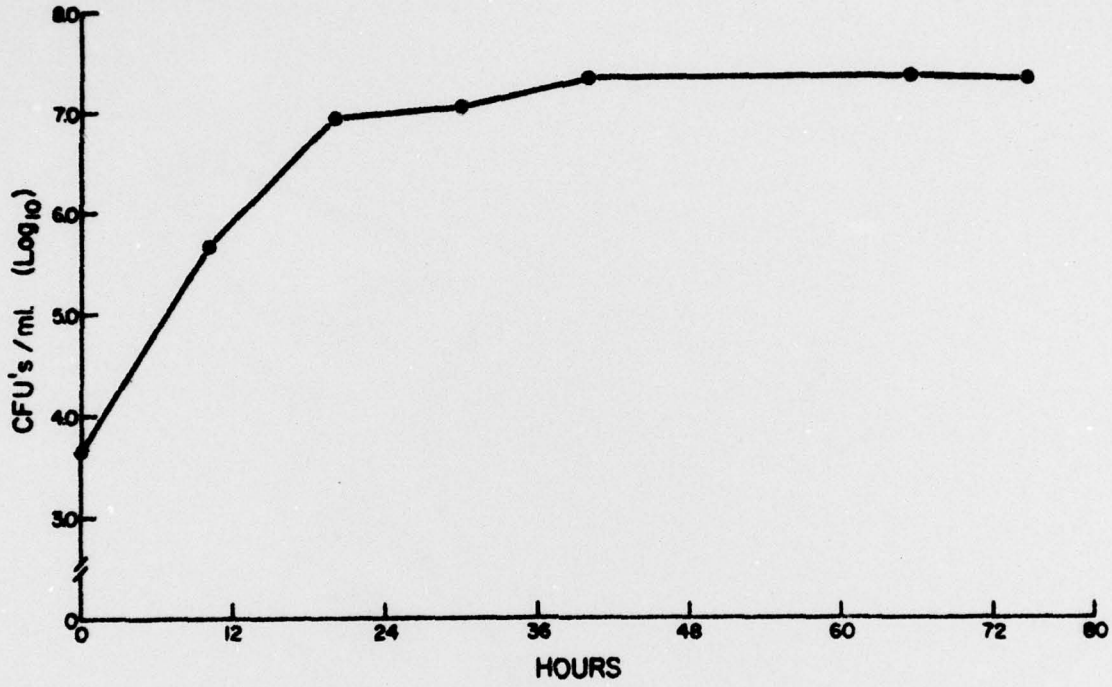

FIG. 7. Growth of A. calcoaceticus During Inactivation of Poliomyelitis virus.

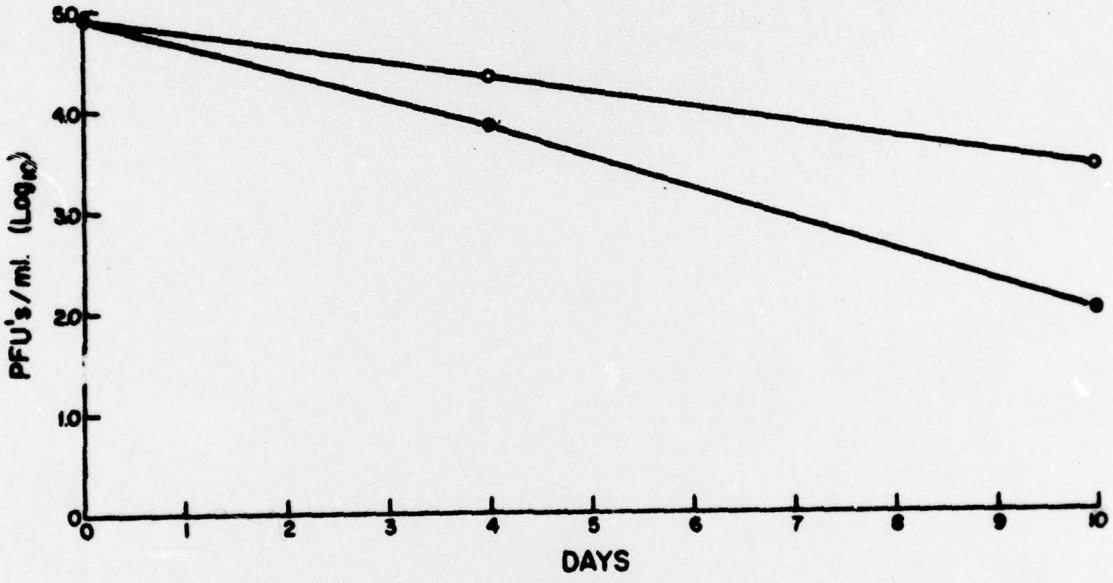

FIG. 8. Survival of Coxsackie B-5 virus in Presence of A. calcoaceticus.

O - A. calcoaceticus present

● - Coxsackie B-5 virus in seawater alone.

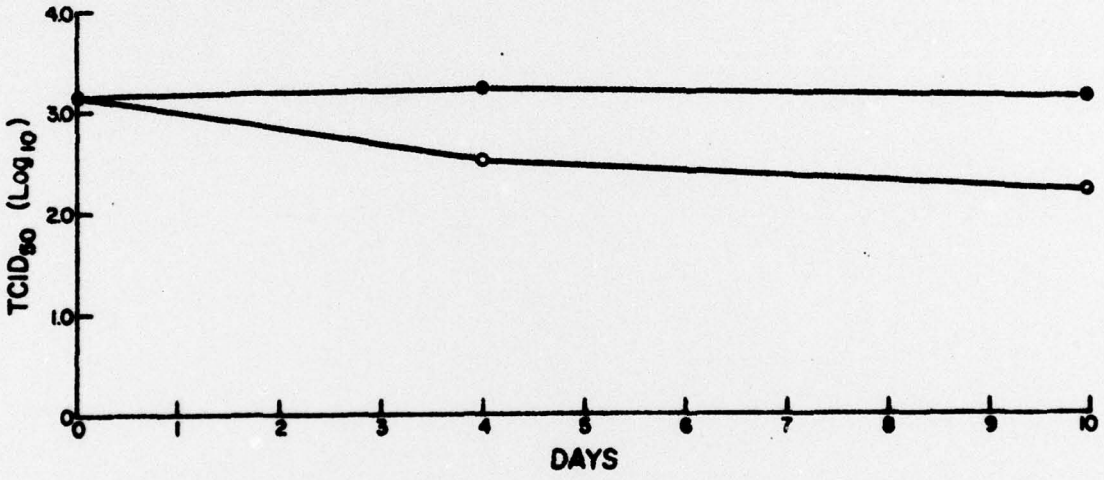

FIG. 9. Survival of H-1 Parvovirus in Presence of A. calcoaceticus.

O - A. calcoaceticus present

● - H-1 parvovirus in seawater alone

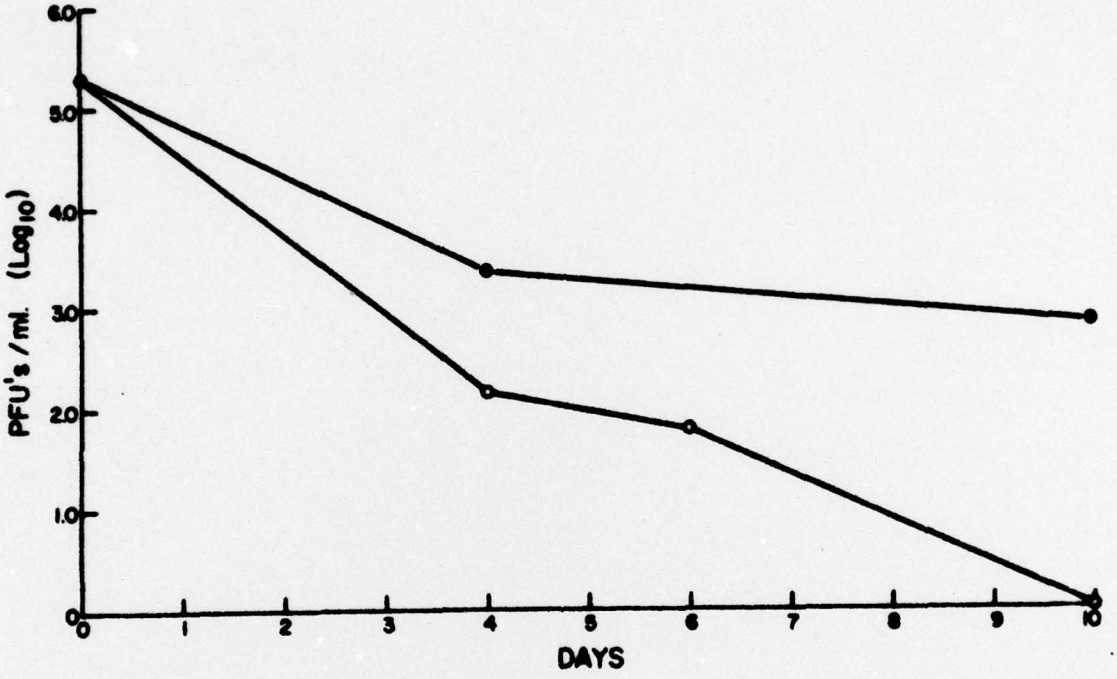

FIG. 10. Slight Inactivation of ECHO-6 Virus in Presence of A. calcoaceticus.

O - A. calcoaceticus present

● - ECHO-6 virus in seawater alone

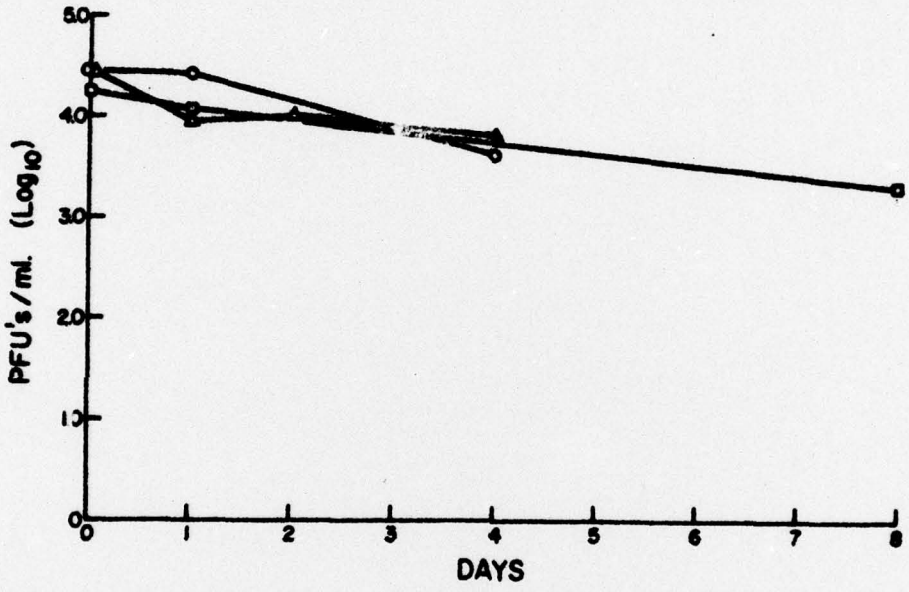

FIG. 11. Survival of Poliomyelitis Virus in Filtrates of Growing A. calcoaceticus cultures.

O - Filtrate taken at 24 hrs. growth

Δ - Filtrate taken at 48 hrs. growth

□ - Filtrate taken at 12 hrs. growth

3. Collaboratorive studies with the Naval Ship Research and Development Center (NSRDC), Annapolis

Procedure

The Pollution Abatement Division of NSRDC is evaluating different equipment prototypes for their effectiveness in shipboard sewage treatment processes. The test sludge that they use consists of a mixture of Chesapeake Bay water and domestic sewage generated on the base. We have been attempting to determine the efficacy of the prototype units in removing human enteroviruses. Samples of the raw sewage entering the units and the effluents leaving the units were tested. Two isolation procedures have been used:

1. Direct inoculation

Samples of sludge were intially sonicated for 5 min in an attempt to release any virus associated with solids. Then the sample was centrifuges at 15,000 x g for 30 mins to sediment the bacteria present. The supernatant fluid was further clarified by passage through a 0.45 µM filter. The filtrate was then inoculated onto a series of BGM monolayers for virus isolation.

2. Polyethylene glycol precipitation

PEG-6000 was added in flake form (50 gms/liter) to the sewage - Bay water mixture and mixed for 14 hr at 4°C. Samples were then centrifuged at 9500 x g for 2 hr. The resultant pellets of precipitated protein were resuspended in buffered saline and sonicated for 5 min. The suspension was then centrifuged at 15,000 x g for 20 min and the supernatant assayed for virus as above.

Results

Numerous attempts to isolate enteroviruses by the above two methods have all been negative. The most likely explanation for these results is that the sewage being used (which comes from a nearby office building) has

variable enterovirus content from day to day and that those viruses which may be present are further diluted out by the addition of the bay water. Experiments are in progress which involve "seeding" the sewage mixture with the f-2 coliphage (a virus similar in size and stability characteristics to the enteroviruses) for the purpose of evaluating the units' ability to remove viruses.

4. Shellfish bacteriological studies

Attention has been focused on an appraisal of methods for coliform enumeration, and determination of the aerobic, heterotrophic bacteria in shellfish of aquatic samples in order to determine the baseline load under natural conditions, for subsequent survival studies of pathogens in dosing experiments using shellfish as the carrier.

Procedure

Shellfish (Crassostrea virginica) were collected from two estuarine sites; viz.; Tolley Point, outside Annapolis harbor, and Eastern Bay of the Chesapeake Bay. Additional sediment samples were collected at Eastern Bay, bottom water was sampled at Tolley Point, and the aquarium water was sampled for coliforms.

Coliform analysis was performed using methods as outlined for the total coliform Most Probable Number (MPN Standard Methods for the Examination of Sea Water and Shellfish, 1970).

Aerobic heterotrophic bacteria were counted on glucose tryptone yeast - extract agar (GYTEA; Divco) and one-third strength Zobell 2216 marine agar (Difco). Plate counts were incubated at 15°C for 7 days.

Shellfish were maintained in a glass aquarium. The animals were collected, rinsed and placed in an aerated estuarine salts solution, in the aquarium. The water was changed every 48 hours. MPN enumeration

was performed on the water upon introduction of the oysters and with every change of the water. After 10 days depuration, the oysters were examined for the presence of coliforms and heterotrophic bacteria.

Shellfish from the Chesapeake Bay were processed within one hour of collection. MPN and aerobic, heterotrophic bacteria counts were determined on homogenized oyster slurry.

With water and sediment samples, coliform analysis was initiated immediately upon collection. Sediment samples were diluted to 10^{-1} (v/v) in 0.5% (w/v) peptone (Difco) and MPN tubes were inoculated with the suspension. Aerobic heterotrophic bacteria plate counts were prepared immediately.

Results

Shellfish: Of the colonies observed on 2216 agar, 50% were gram-negative rods, of which, 90% were psendomonads. Using conventional taxonomic tests, Pseudomonas aerugenosa was identified in 70% of the samples. It is interesting to note that in Eastern Bay, P. aerugenosa occasionally predominates in the oyster slurry. Pseudomonas spp. have been observed in two shellfish samples, while the corresponding water and sediment appeared negative for pseudomonads.

Forty-per cent of the colonies were chromogenic.

Plate counts on the 2216 agar were at least 100% greater than counts on GTYEA, and also displayed greater species diversity as determined by examination of colonial and micro-morphology.

Nearly half the colonies were not "Eubacteria." These included myxobacteria (three samples), flexibacteria (three samples), arthrobacteria (four samples), caulobacteria (one sample), hyphomicrobia (one sample),

actinomycetes (three samples), some vibrio-like organisms, and Bacillus spp. (a minority, but uniformly present). There were other morphological types observed which have yet to be described.

Estuarine shellfish contained total aerobic heterotrophic bacteria counts of between 10^4 and 10^5/gm. The heterotrophs in whole oyster homogenate are generally the same as those in surrounding water and sediment. Oysters may concentrate these organisms, however they do not eliminate them quickly. MPN values ranged from less than six, to as high as 330 coliforms per 100 gm of oyster. Crassostrea virginica was not found to maintain a coliform population.

Sub-estuaries with non-point sources of coliform input, deliver indicator bacteria to the Chesapeake Bay at levels which are highest in the late summer and autumn. This trend has been previously established, but the levels may be exaggerated by additional effluents. At Tolley Point where effluents are present, shellfish concentrated the indicator bacteria.

With the baseline data obtained, it is now possible to carry out survival studies using shellfish as the carrier.

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